PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Robert Michael Roberts *et al.*

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For: COMPOSITIONS AND METHODS FOR EARLY PREGNANCY DIAGNOSIS

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37 C.F.R. 1.8

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Robert E. Hanson

**DECLARATION OF JONATHAN A. GREEN UNDER 37 C.F.R. § 1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

I, JONATHAN A. GREEN, HEREBY DECLARE AS FOLLOWS:

1. I am a co-inventor of the subject matter disclosed and claimed in the above-referenced patent application.

2. I am currently employed by The University of Missouri as an Assistant Professor. I hold a Ph.D. in Biochemistry from the University of Missouri. I have been conducting research in the area of biochemistry and reproductive biology since 1991.

3. I understand that the Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application has rejected the claims as not being supported by

adequate information in the specification to show that pregnancy associated antigens (PAGs) are present early in pregnancy and absent about two months post-partum.

4. Therefore, I am providing the present Declaration to submit further data that demonstrates the isolation and use of monoclonal antibodies that detect PAGs disclosed in the above-referenced patent application. The data presented below demonstrates that the specification teaches those of ordinary skill in reproductive biology how to make and use PAGs that are present early in pregnancy and absent about two months post-partum without undue experimentation.

5. *Preparation of proteins for immunizations:* Two stages of placental tissue were used to produce monoclonal antibodies specific for PAGs. The earliest stage consisted of secretory proteins from an explant culture of pools of day 24-34 trophoblast. The other source of protein was obtained from explant culture of cotyledons from ~ day 80 pregnant cows collected from a local slaughterhouse. PAGs were enriched from each tissue source by affinity purification with the aspartic proteinase inhibitor, pepstatin A as described below.

Whole trophoblast (d 24-34) or cotyledons (d 80) were gently separated from the uterine caruncles and the rest of the extra-embryonic membranes as cleanly as possible. The tissues were cut into ~2 $mm^3$ pieces, washed three times in Dulbecco's Modified Eagle's medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.5μg/ml) and cultured in the same medium at 37°C in 5% CO2:95% air for 12 h. After the incubation, the tissue and medium were separated by centrifugation and the supernatant was stored at -20°C until used. Proteins within the conditioned medium were thawed, then precipitated by sequential addition of $(NH_4)_2SO_4$ to 20%, 40%, and 75% saturation. The pellets were resuspended, dialyzed against 20 mM Tris, pH 8.0, and the presence of immunoreactive PAG was determined by immunoblotting with a rabbit anti-oPAG-1 antiserum raised against recombinant oPAG-1 produced in bacteria (unpublished results). The 40-75% fraction contained the most PAG.

The 40-75% fraction was dialyzed extensively against 20 mM Tris pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.02% (w/v) sodium azide, 20 μM PMSF, 1 mM 2-mercaptoethanol. Insoluble material was cleared by centrifugation and the supernatant was applied to a column containing 100 ml of beaded pepstatin-A agarose (4% cross-linked, Sigma, St Louis, MO)

equilibrated in the same buffer. The column was washed with 10 column volumes of loading buffer, then washed with an additional 10 column volumes of 20 mM Tris pH 7.0, 1 M NaCl, 1% Triton X-100, 1 mM EDTA, 0.02% (w/v) sodium azide, 20 μM PMSF, 1 mM 2-mercaptoethanol. Proteins bound to the column were eluted by increasing the pH of the buffer in a stepwise fashion (pH 8.0, pH 9.5 and pH 10.5). Elution fractions were collected and analyzed by dot blot to identify those fractions containing immunoreactive PAG. Those fractions from each stepwise elution that contained PAG were pooled and the concentration of PAG in the collected fractions was determined by Bradford assay.

Cotyledonary extracts from d150 pregnancies were purified by pepstatin as described above for the production of anti-PAG polyclonal antiserum to be used as the detection reagent in the sandwich ELISA.

6. *Polyclonal antibody production:* Polyclonal antibodies were produced in rabbits by immunization of rabbits with PAG purified by pepstatin from day 150 pregnant cotyledons. Briefly, pooled fractions eluted from the column (0.25 mg) were mixed with 0.5 ml of Freund's complete adjuvant, and injected s.c. at multiple sites along the back of a New Zealand White rabbit (Harlow, E., and Lane, D. (1988). Antibodies: A laboratory manual). The rabbit was boosted with antigen at 4- to 5-wk intervals with 0.1 mg of the PAG-enriched fractions in Freund's incomplete adjuvant. Blood was collected from the central ear vein 12–14 days after each booster injection and allowed to clot at 4°C overnight, and the serum was stored at -20°C. All procedures were carried out with the approval of the University of Missouri Animal Care and Use Committee (protocol #1292).

7. *Hybridoma production and screening:* For monoclonal antibody production, two mice were immunized with PAG-enriched fractions (75μg per mouse) from both the day 24-34 and the day 80 isolations by mixing the preparations (85% from the day 80 material and 15% from the day 24-34 material). The mice were boosted 4 weeks later with 42 μg PAG per mouse. TiterMax® adjuvant (TiterMax, Inc., Norcross, GA) was used for both the immunization and the booster injection. The production of the hybridomas was adapted from standard protocols (De St. Groth, S. F., and Scheidegger, D. (1980). Production of monoclonal antibodies: Strategy and

tactics. J Immunol Methods *35*, 1-21). Mouse myeloma cells were fused with the murine spleen cells in PEG, then plated on macrophage-seeded 96-well culture plates in HAT-HL-1 medium (Sigma, St Louis, MO; Bio-Whittaker, Walkersville, MD). Preliminary ELISAs (direct and a double antibody sandwich) were run after 10 days in culture. In the sandwich ELISA a 96-well plate (CoStar, Corning, NY) was coated with a sheep anti-mouse (Jackson ImmunoResearch, West Grove, PA ) orienting trap, and then the hybridoma culture medium was added. In the direct ELISA, the plate was coated with the antigen first. The same PAG mixture that was used to immunize the mice was used as antigen in both assays. After two rounds of selection 132 positives were left: 57 of these were positive in both assays, 61 were positive only in the sandwich method, 14 in the direct method only. All positives were tested for cross-reactivity with BSA, bovine endometrial extracts, non-pregnant heifer serum and cathepsin D to rule out false positives. Five lines that exhibited reactivity toward these proteins were not characterized further. A total of 127 positive colonies remained. Finally, the 127 positives were tested for reactivity against the day 24-34 PAG only. Fifty of the 127 positives exhibited the greatest reactivity toward the d 24-34 PAG. Ten of these were selected for further study. Lines were obtained by limited dilution, expanded, and frozen. Three of the lines (L4, A6, J2) grew well in culture and were used as the basis for the assay.

8. *IgG production and isolation:* Large scale production of IgG was achieved by thawing frozen hybridoma cell stocks, growing them in the presence of mouse macrophage, and collecting medium from expanded cultures (Harlow and Lane, 1988). Concentrated medium was concentrated by Ultrafiltration and dialyzed against 1.5 M NaCl, 100 mM glycine, pH 9.5 and loaded onto a protein A-Sepharose (Pierce) column, equilibrated in the same buffer, by using a BioLogic FPLC (Bio-Rad, Hercules, CA). The column was washed with five volumes of loading buffer and bound immunoglobulin was eluted with 100 mM sodium citrate, pH 3.0. Immunoglobulin present in the eluted fractions was quantified by Bradford assay with rabbit IgG (Sigma) as the standard. Polyclonal immunoglobulins in rabbit serum were isolated in the same manner as for the immunoglobulins secreted by the hybridomas.

9. *Indirect sandwich ELISA for detection of PAG in heifer and cow serum:* Serum was collected from heifers and cows at the time of standing estrus, on day 15 after insemination, daily from days 22 to 28 post-AI, and weekly throughout the remainder of pregnancy and for several weeks after parturition. The samples analyzed in this study were only from those animals (n=42) that maintained a pregnancy and successfully delivered a live calf.

An ELISA was used employing a mixture of monoclonal antibodies (L4, J2, A6) to trap PAG in the wells of a 96-well ELISA plate. An anti-PAG polyclonal antiserum raised in rabbits was used to bind to the immobilized PAG and the complex was detected by using an alkaline phosphatases-conjugated anti-rabbit antibody. The trapped monoclonal antibodies were oriented in the wells by the use of 1μg of sheep anti-mouse Fc (Jackson ImmunoResearch, West Grove, PA) that had been incubated in the wells in the presence of 0.1 M sodium bicarbonate, pH 9.5 overnight at 4C. The anti-mouse antibody was removed by washing the plates 3 times with 0.15 M NaCl, 0.05% Tween-20 with a 96-well plate washer (ELx405, BioTek, Winooski, VT). The wells were filled with blocking solution (2% ovine serum albumin, 1% nonfat dry milk) and incubated at RT for 1 hour. The blocking solution was removed and 100 μL of a monoclonal mixture (500 ng/mL each of monoclonals A3, J2, L4, diluted in TBST) was added to each well and incubated at RT for 1 hour. The antibody solution was removed, the wells were washed and 50 μL of TBST was added to each well to keep them moist. Next, 100 μL of pregnant bovine serum or serially diluted PAG standards (in non-pregnant heifer serum) were added to duplicate wells. Non-pregnant heifer serum alone was included as a blank. The plates were incubated O/N at 4C. The following day, the plates were washed and 100 μL of a 20 μg/ml anti-PAG polyclonal IgG was added to each well and incubated at RT for 1 hour. The plate was washed and 100 μL of AP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), diluted 1:2000, was added to the wells and incubated at RT for 20-30 minutes. The plate was washed and 100 μL of 1 mg/ml PNPP (Sigma, St. Louis, MO) was added to each well. An EL808 plate reader (Bio-Tek) was used to measure the absorbance at 405 nm in the wells.

10. *Expression profiles of L4, A6 and J2 antibodies:* The results of the ELISA are shown in the four graphs in **Exhibit 1**. The "error bars" are the standard deviation of the average PAG concentrations at each stage. The graphs showing post-partum values are based on 39 of the 42

animals as post-partum sera was not available for 3 of the animals. In the assay, the criterion for designating an animal pregnant was 1 ng/ml. In the assay, nearly all the animals had PAG concentrations below this value by 6 weeks post-partum. Only two animals had PAG values above the threshold by week six. One did not fall below the threshold until post-partum week 10 and the other never had PAG immunoreactivity in the assay below 3.4 ng/ml, even in blood samples taken prior to AI and at early stages of pregnancy (e.g. day 14). These animals were indicated to be outliers and non-indicative of typical cows.

The Ln PAG v. week post-partum plot was calculated from all post-partum samples except the "high-background" animal, which was excluded as this animal's samples deviated from linearity in the PP week 7 and 8 values. The plot illustrates that PAG values fall below the 1ng/ml threshold by week 6 post partum in the assay. Based on the line equation, it also indicates that the half-life of those PAG in maternal serum recognized by the L4, A6 and J2 monoclonals is 4.3 days. This half-life is considerably shorter than that reported by others using bovine PAG-1 RIA (e.g., Kiracofe *et al.*, 1993, *J Anim Sci* 71:2199-2205; Melo de Sousa et al., 2002, *Theriogenology* 8706:1-12). In these reports, PAG-1 half-life was reported to vary from 8.4 to 10.1 days. Based on these results, it was clear that the PAGs detected by these antibodies were absent at approximately two-months after pregnancy in all but a statistically insignificant number of outlier cows.

11. *Confirmation of PAGs detected by L4, A6 and J2 antibodies:* Studies are currently being carried out to identify the PAGs recognized by the L4, A6 and J2 antibodies. MALDI-TOF was performed on purified proteins from two separate placenta extracts (obtained from cotyledons of a 16 in. crown-rump fetus and an 18 cm crown-rump fetus) to identify the PAGs bound by the A6 and L4 antibodies; the results from the J2 purification are not yet available. For the A6 monoclonal, each purification attempt lead to the isolation of two distinct proteins (~52kDa and ~75kDa). The mass spectrometry analysis revealed matches for bovine PAG4, PAG6, PAG7 and PAG16, indicating that these PAGs are targets of the A6 monoclonal.

For the L4 monoclonal, results from the first purification (16 in. crown-rump fetus) revealed matches with PAG20. The second purification (18 cm crown-rump fetus), revealed matches with PAG20 and PAG6. These results were not as robust as those obtained from the A6 purification. The first purification only matched 5 of 26 peptides submitted for analysis. The

second purification only matched 8 of 57 peptides submitted (5 hit PAG20, 5 hit PAG6; 2 of the peptides were in common with PAG6 and PAG20). An attempt will be made to improve the results by deglycosylating the proteins to a greater extent and resubmitting them for analysis. However, the fact that PAG20 came up in the two separate purification attempts indicates that it is a target of L4.

12. *Additional studies to determine PAGs bound by L4, A6 and J2 monoclonal antibodies:* A further study was carried out to identify PAGs detected by the L4, A6, and J2 antibodies described above as follows:

One mg of each purified mAb was first crosslinked to 2 mL of matrix in the ImmunoPure Protein A IgG Plus Orientation kit (Pierce Biotechnology, Inc. Rockford, IL, USA) by following the manufacturer's instructions. Cotyledonary extracts were collected from 18 cm and 40 cm crown-rump bovine fetuses, dialyzed against 2000 volumes of binding buffer and 100 mg of total protein from each extract was applied separately to each matrix. The columns were washed in binding buffer until the absorbance of the flow-through at 280 nm was at baseline. The bound protein was eluted from the column with 20mM sodium formate, pH 2.8. The eluted proteins were subjected to SDS-PAGE followed by in-gel trypsin digestion, reduction and alkylation of cysteines. The masses of the resulting peptides were then determined by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager-DE™ PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA). The monoisotopic masses in the acquired spectra were used for searching against a nonredundant translated mammalian sequence database (NCBInr) by using the Protein Prospector MS FIT program (http://prospector.ucsf.edu/).

The A6 monoclonal antibody exhibited the greatest ability to bind PAG in the placental extracts. The eluted material migrated at three distinct relative molecular mobilites on SDS-PAGE: 55,000, 65,000 and 75,000. Peptide mass fingerprinting revealed that the 75kDa band consisted predominantly of PAG7 and PAG6 and the 55kDa band consisted predominantly of PAG16 and PAG4, although weak matches were also observed for PAG17, PAG20 and PAG21 in this band (**Exhibit 2, Table 1**). The 65kDa band did not produce many peptides amenable to fingerprinting, but the few that were produced were found to match PAG7.

The yields from the J2 and L4 affinity columns were not as robust as those from the A6 matrix, however, both did permit the purification of a ~70,0000 Da protein. The J2-purified protein did not produce many tryptic fragments, but those that were produced matched PAG20 (**Exhibit 2, Table 2**). The L4-purified protein was more easily digested and produced numerous masses for fingerprinting. The main PAG isolated from the extracts was PAG21 although other PAGs (PAG17, PAG16 and PAG20) were clearly represented, albeit at lower concentrations (**Exhibit 2, Table 3**). The A6 and L4 antibodies bound PAG17 with lower affinity, but the results confirmed the undetectability of this PAG at about two-months post-partum. The major PAGs recognized by each of the monoclonal antibodies (and their relationship to other bovine PAGs) are indicated in the neighbor-joining tree (**Exhibit 3**).

13. *Analysis of PAG presence in bovine milk, urine and saliva:* An analysis was carried out to determine whether PAGs could be detected in the milk, urine and saliva of pregnant bovine animals. The samples were collected from each of the animals prior to entering the milking parlor. Milk samples (~30mL) were collected after cleaning the teats. The samples were centrifuged to remove the lipid and the remaining cleared solution was harvested and frozen at -20°C for storage. For collection of urine samples, animals were induced to urinate by stimulation of the perineum and vulval tip. Urine was collected in a 50mL container and centrifuged to remove debris. The supernatant was harvested and frozen until assayed. Saliva was collected by sweeping a cotton swab inside the mouth alongside the lower jaw and dragging the saliva into a collection tube. 2-10 mL of saliva could be collected in this way. These samples were centrifuged and the supernatant was used in ELISA assays. Some of the animals were not available, or sufficiently cooperative, for saliva extraction and thus saliva samples were not available for all of the animals tested.

ELISA was carried out on the milk, urine and saliva samples by using the L4, A6 and J2 monoclonal-based sandwich ELISA as described above for serum samples. A polyclonal-based anti-PAG ELISA was also utilized employing the polyclonal antibodies produced according to paragraph 6 above. As shown in **Exhibit 4**, four of seven pregnant cows tested showed presence of PAG in milk in both assays. In the polyclonal assay, five of the seven pregnant animals exhibited reactivity in urine, whereas the monoclonal antibodies detected PAG in two of the

seven pregnant urine samples. Saliva samples were available for only five of the pregnant animals and all showed the presence of PAG in the polyclonal-based assay, while three of the pregnant saliva samples were positive in the monoclonal-based assay. **(Exhibit 4)**. Western blot analysis was also carried out to analyze the presence of PAGs in milk and urine. The polyclonal anti-bovine PAG antibodies detected PAG in milk, but not urine. A normal rabbit serum control did not exhibit any reactivity **(Exhibit 5)**.

The results demonstrated that, in addition to serum, PAG is present in other biological samples from pregnant bovine animals including saliva, milk and urine.

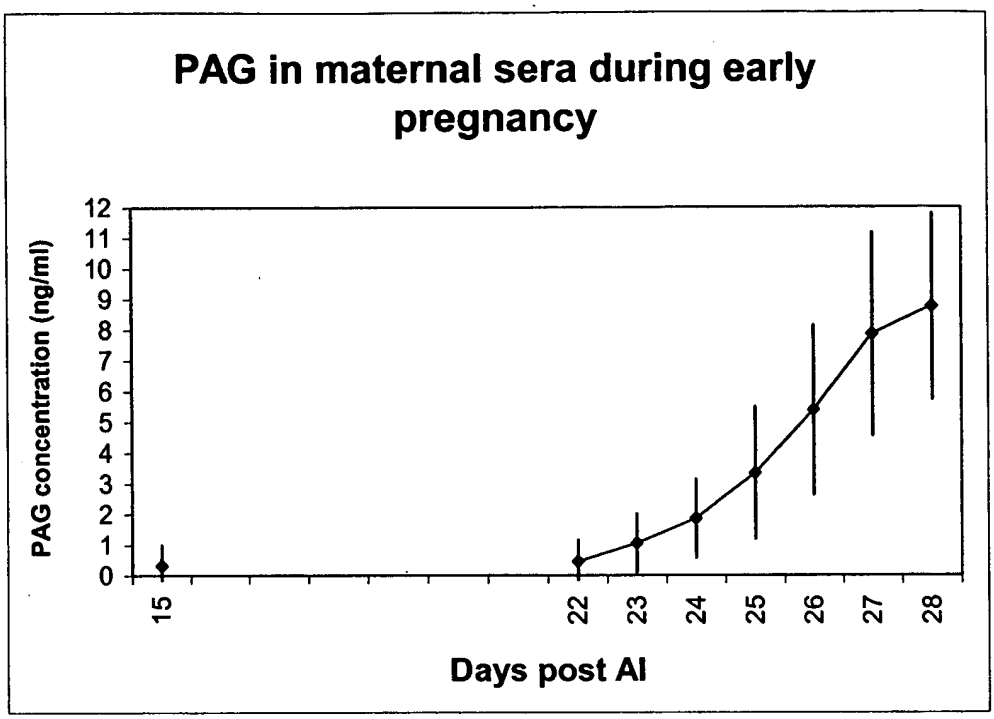
14. The result of the studies with the L4, A6 and J2 antibodies demonstrated that PAGs 4, 6, 7, 16, 17, 20 and 21 are undetectable about two-months post-partum and that antibodies for these PAGs may be used in assays for the detection of pregnant bovine animals. Based on the results, a person of ordinary skill in the field of reproductive biology would be able to generate similar results using the teaching in the specification. The results further demonstrate that one of skill in the art as of the filing date of the patent application would have been able to, without undue experimentation, detect pregnancy in a bovine animal using the method of claim 182 of the above-reference patent application. Specifically, the descriptions in the patent application enable a person of ordinary skill in reproductive biology to, without more than routine experimentation, (a) obtain a sample from a bovine animal; and (b) detect at least one pregnancy associated antigen (PAG) in the sample that is present early in pregnancy and is undetectable at about two months post-partum; wherein detection of the PAG indicates that the animal is pregnant.

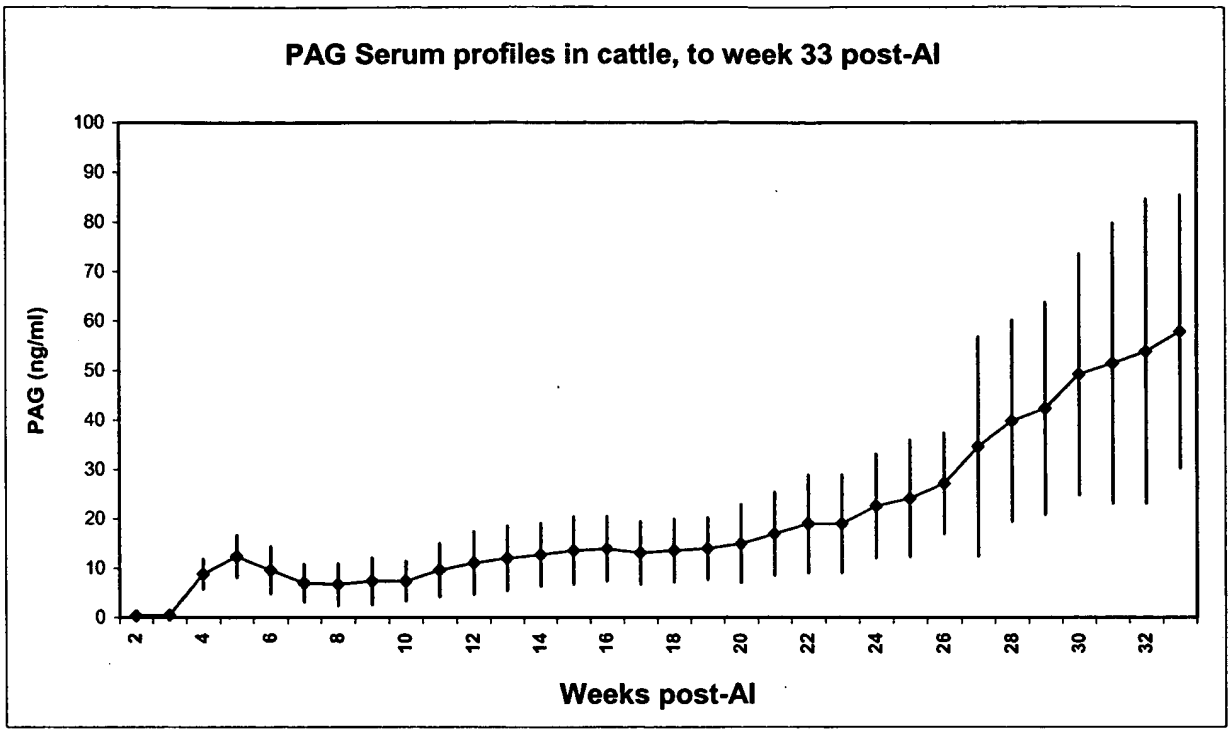
15. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

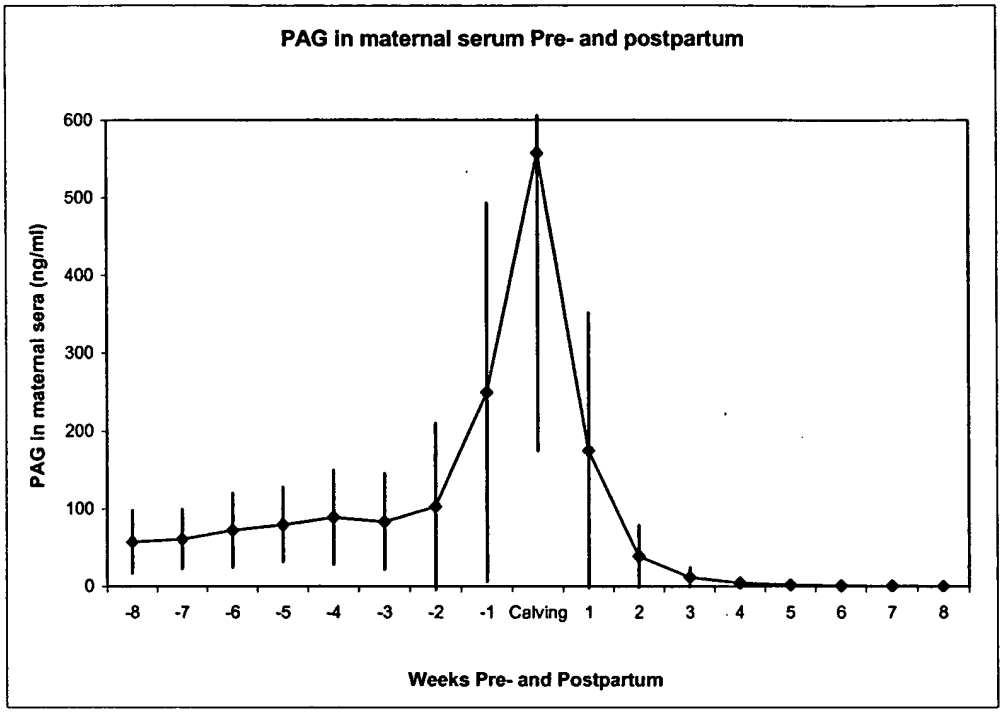
14 September 2004

Date

Jonathan A. Green







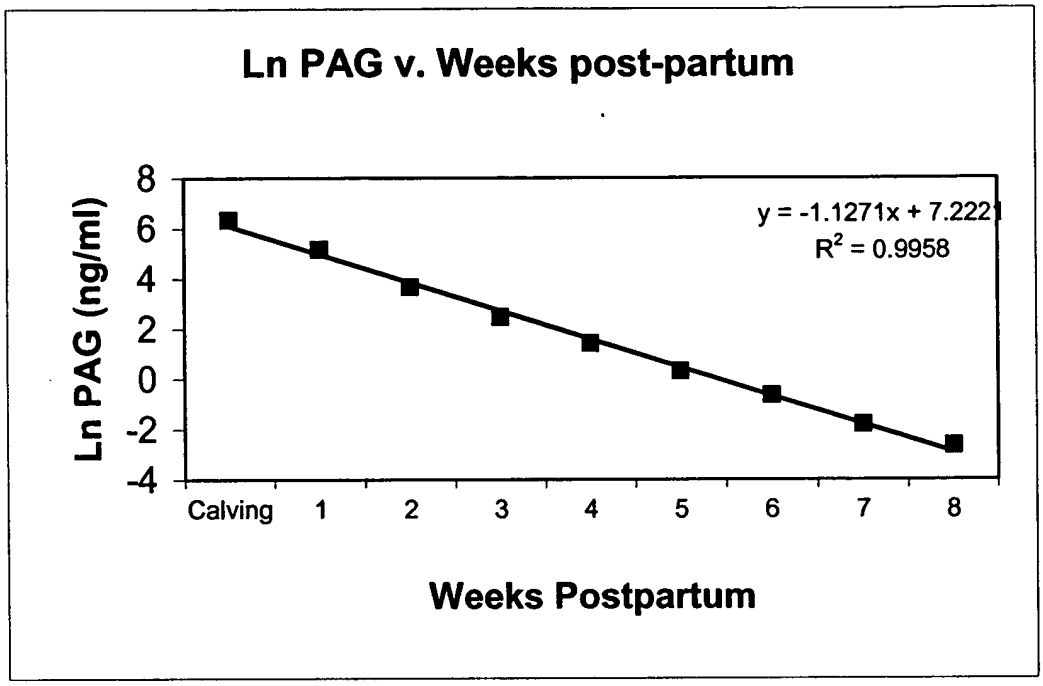


Table 1. Assignment of tryptic digest fragments of PAG affinity purified with the A6 monoclonal antibody.

75 kDa protein

1. BOS TAURUS. (AF020509) pregnancy-associated glycoprotein 7.

| m/z submitted | MH+ matched | Delta ppm | Fragment | Database Peptide Sequence | Modifications |
|---|---|---|---|---|---|
| 529.3440 | 529.3462 | -4.1681 | 335-339 | (R)IGLAR(R) | |
| 727.4410 | 727.4466 | -7.7652 | 250-256 | (R)IIGATPR(G) | |
| 765.4290 | 765.4259 | 4.0326 | 130-136 | (R)IIYGSGR(M) | |
| 871.4980 | 871.5001 | -2.4566 | 243-249 | (R)LIDNIQR(I) | |
| 1046.5410 | 1046.5311 | 9.4534 | 323-330 | (R)LYFSVFDR(G) | |
| 1238.6130 | 1238.6203 | -5.9199 | 137-147 | (R)MNGVIAYDTVR(I) | |
| 1254.6170 | 1254.6152 | 1.3971 | 137-147 | (R)MNGVIAYDTVR(I) | 1Met-ox |
| 1479.8120 | 1479.7994 | 8.542 | 30-42 | (K)TLSGKNMLNNFLK(E) | |
| 1488.7300 | 1488.7236 | 4.3312 | 323-334 | (R)LYFSVFDRGNDR(I) | |
| 1522.8030 | 1522.7906 | 8.1633 | 310-322 | (R)STESWVLGEVFLR(L) | |
| 1678.8830 | 1678.8917 | -5.1701 | 309-322 | (R)RSTESWVLGEVFLR(L) | |
| 2509.2380 | 2509.2251 | 5.1429 | 148-170 | (R)IGDLVSTDQPFGLSVEEYGFAHK(R) | |
| 2548.1970 | 2548.1772 | 7.7798 | 218-242 | (R)EVIACSEGCAALVDTGSSNIQGPGR(L) | |
| 2665.3420 | 2665.3282 | 5.9256 | 148-171 | (R)IGDLVSTDQPFGLSVEEYGFAHKR(F) | |

2. BOS TAURUS. (AF020508) pregnancy-associated glycoprotein 6.

| m/z submitted | MH+ matched | Delta ppm | Start | Peptide Sequence | Modifications |
|---|---|---|---|---|---|
| 529.344 | 529.3462 | -4.1681 | 373-377 | (R)IGLAR(A) | |
| 933.434 | 933.447 | -13.9726 | 362-368 | (R)YFSVFDR(G) | |
| 1211.569 | 1211.5632 | 4.8269 | 336-344 | (R)CYTAFKEHR(F) | |
| 1237.585 | 1237.5731 | 9.6461 | 126-135 | (K)TFRITYGSGR(M) | 1PO4 |
| 1375.642 | 1375.6395 | 1.826 | 362-372 | (R)YFSVFDRGNDR(I) | |
| 1439.807 | 1439.7144 | 64.3292 | 114-125 | (R)HHQSSTFRPTNK(T) | |
| 1760.849 | 1760.839 | 5.6833 | 212-227 | (K)DKQEGSVVMFGGVDHR(Y) | |
| 1762.88 | 1762.9049 | -14.1511 | 147-162 | (R)IGDLVSTDQPFGLCLK(D) | |
| 1771.878 | 1771.8907 | -7.1546 | 196-211 | (K)NEGAISEPVFAFYLSK(D) | |
| 1776.849 | 1776.8339 | 8.4942 | 212-227 | (K)DKQEGSVVMFGGVDHR(Y) | 1Met-ox |
| 2013.066 | 2013.0697 | -1.8401 | 194-211 | (K)LKNEGAISEPVFAFYLSK(D) | |
| 2297.165 | 2297.1488 | 7.07 | 147-167 | (R)IGDLVSTDQPFGLCLKDSGFK(G) | |
| 2300.223 | 2300.2138 | 3.9986 | 266-287 | (K)ALVDTGTSDIVGPSTLVNNIWK(L) | |

55 kDa protein*

1. BOS TAURUS. (AF192333) pregnancy-associated glycoprotein-16.

| m/z submitted | MH+ matched | Delta ppm | Start | Peptide Sequence | Modifications |
|---|---|---|---|---|---|
| 529.3000 | 529.3462 | -87.2895 | 374-378 | (R)IGLAR(A) | |
| 727.4150 | 727.4466 | -43.5066 | 289-295 | (K)LIGATPR(G) | |
| 1046.5000 | 1046.5311 | -29.7237 | 362-369 | (R)LYFSVFDR(G) | |
| 1167.5590 | 1167.5911 | -27.4764 | 242-251 | (R)AGDWIVHVDR(I) | |
| 1389.6420 | 1389.6585 | -11.8799 | 216-228 | (R)EGSVVMFGGVDHR(Y) | |
| 1405.6410 | 1405.6534 | -8.8383 | 216-228 | (R)EGSVVMFGGVDHR(Y) | 1Met-ox |
| 1422.6550 | 1422.6476 | 5.1946 | 335-345 | (R)GYCYTAFKEQR(V) | |
| 1436.6760 | 1436.7286 | -36.637 | 115-126 | (R)HFQSSTFRPTTK(T) | |
| 1488.6990 | 1488.7236 | -16.492 | 362-373 | (R)LYFSVFDRGNDR(I) | |
| 1516.7570 | 1516.695 | 40.8992 | 115-126 | (R)HFQSSTFRPTTK(T) | 1PO4 |
| 1522.7750 | 1522.7906 | -10.224 | 349-361 | (R)STESWLLGDVFLR(L) | |
| 1621.8770 | 1621.7641 | 69.6458 | 43-54 | (K)EHPYRLSHISFR(G) | 1PO4 |
| 1650.9090 | 1650.9008 | 4.9741 | 229-241 | (R)YYKGELNWVPLIR(A) | |
| 1739.8990 | 1739.8982 | 0.4814 | 113-126 | (R)FRHFQSSTFRPTTK(T) | |
| 1859.9220 | 1859.875 | 25.247 | 216-231 | (R)EGSVVMFGGVDHRYYK(G) | 1Met-ox |
| 2012.0830 | 2012.0857 | -1.3362 | 195-212 | (K)LKNQGAISDPIFAFYLSK(D) | |

2. BOS TAURUS. (AF020506) pregnancy-associated glycoprotein 4

| m/z submitted | MH+ matched | Delta ppm | Start | Peptide Sequence | Modifications |
|---|---|---|---|---|---|
| 842.477 | 842.4323 | 53.0192 | 252-257 | (R)ISMKRK(V) | 1PO4 |
| 1046.5 | 1046.5311 | -29.7237 | 362-369 | (R)LYFSVFDR(R) | |
| 1088.517 | 1088.5376 | -18.9692 | 127-136 | (R)TFSITYGSGR(I) | |
| 1244.612 | 1244.6388 | -21.5 | 126-136 | (R)RTFSITYGSGR(I) | |
| 1251.674 | 1251.7061 | -25.6538 | 137-147 | (R)IEALVVHDTVR(I) | |
| 1346.686 | 1346.7432 | -42.4913 | 323-334 | (R)VPGQAYILKDSR(G) | |
| 1488.699 | 1488.8035 | -70.2132 | 115-126 | (R)HLESSTFRLSRR(T) | |
| 1622.853 | 1622.8655 | -7.6811 | 318-331 | (K)SNNYRVPGQAYILK(D) | |
| 1639.898 | 1639.8321 | 40.1684 | 323-336 | (R)VPGQAYILKDSRGR(C) | 1PO4 |
| 1981.032 | 1981.0255 | 3.257 | 318-334 | (K)SNNYRVPGQAYILKDSR(G) | |
| 2013.061 | 2013.0697 | -4.3238 | 195-212 | (K)LKNEGAISEPVFAFYLSK(D) | |
| 2191.087 | 2191.0606 | 12.0431 | 343-361 | (K)GHQQSSSTEMWILGDVFLR(L) | |
| 2286.211 | 2286.1982 | 5.6197 | 267-288 | (K)ALVDTGSSDIVGPSTLVNNIWK(L) | |
| 2413.262 | 2413.2404 | 8.9708 | 195-215 | (K)LKNEGAISEPVFAFYLSKDER(E) | |
| 2547.267 | 2547.2332 | 13.2738 | 35-54 | (K)NMLNNFVKEHAYRLSQISFR(G) | 1PO4 |
| 2700.406 | 2700.4653 | -21.9466 | 172-196 | (R)FDGVLGLSYTNISPSGAIPIFYKLK(N) | |
| 3089.662 | 3089.5981 | 20.6669 | 289-317 | (K)LIGATPQGSEHYVSCSAVNSLPSIIFTIK(S) | |

* Weaker matches were observed for PAG-17, PAG-20 and PAG-21

**Table 2. Assignment of tryptic digest fragments of PAG affinity purified with the J2 monoclonal antibody.**

BOS TAURUS. (AF192337) pregnancy-associated glycoprotein-20

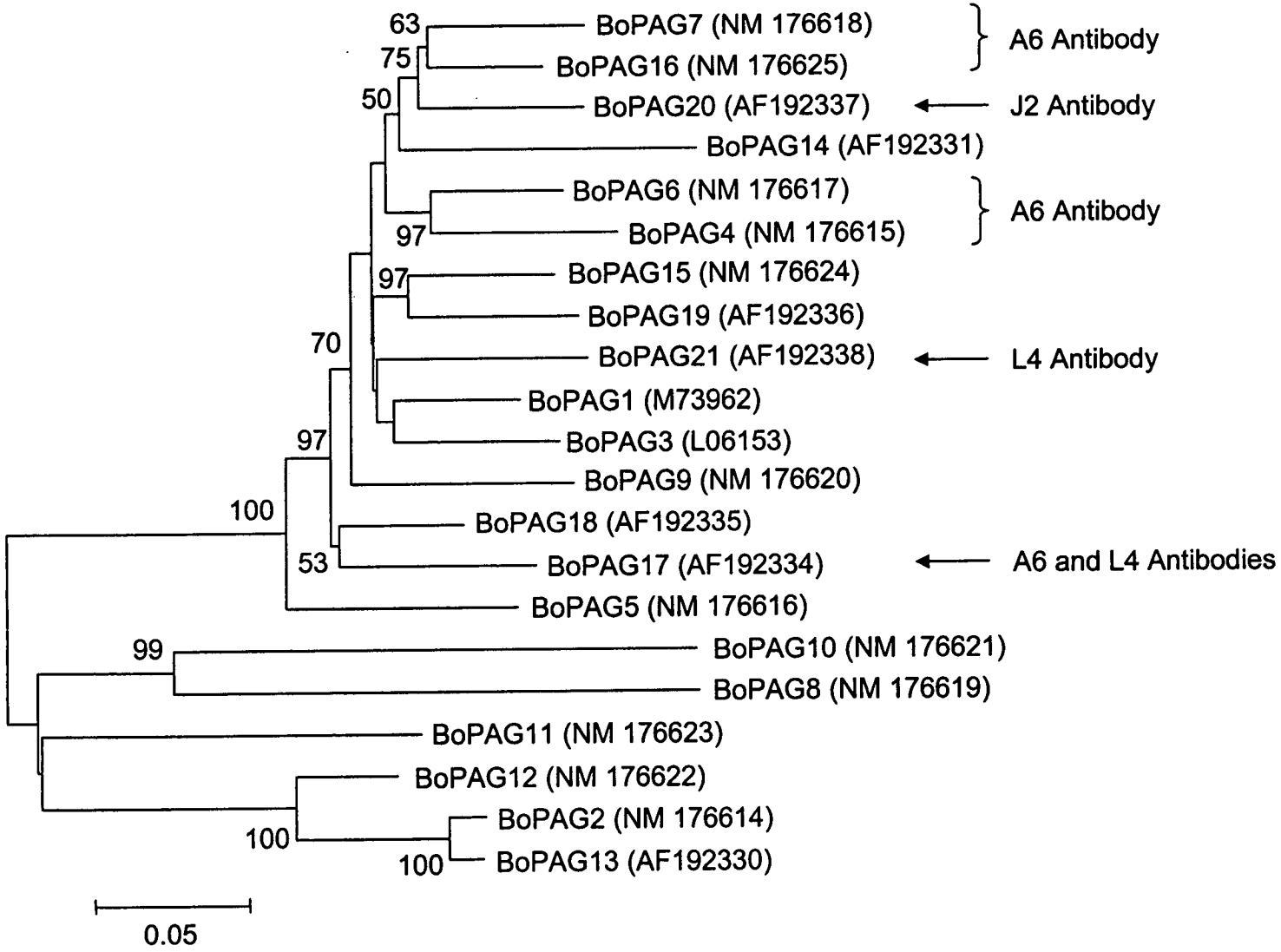
| m/z submitted | MH+ matched | Delta ppm | Start | Peptide Sequence | Modifications |
|---|---|---|---|---|---|
| 1046.586 | 1046.5311 | 52.4526 | 362-369 | (R)LYFSVFDR(G) | |
| 1637.861 | 1687.8607 | 0.2052 | 29-42 | (R)KTLSGKNMLNNFLK(E) | 1PO4 |
| 1789.975 | 1789.9271 | 26.7724 | 123-138 | (R)LTNKTFGITYGSGRMK(G) | 1Met-ox |
| 1794.882 | 1794.8039 | 43.5329 | 217-231 | (K)GSVVMFGGVDHRYYK(G) | 1PO4 |
| 1895.032 | 1895.0505 | -9.7565 | 3-18 | (K)WLVLLGLVAFSECIFK(I) | |
| 1927.919 | 1927.9264 | -3.8439 | 327-342 | (R)AYVLKDFTGNCYTTFK(E) | |
| 2221.268 | 2221.1366 | 59.1792 | 117-136 | (R)QSSTFRLTNKTFGITYGSGR(M) | |

**Table 3. Assignment of tryptic digest fragments of PAG affinity purified with the L4 monocional antibody.**

BOS TAURUS. (AF192338) pregnancy-associated glycoprotein-21 .

| m/z submitted | MH+ matched | Delta ppm | Start | Peptide Sequence | Modifications |
|---|---|---|---|---|---|
| 842.557 | 842.5464 | 12.625 | 282-288 | (K)LVNKIQK(L) | |
| 965.513 | 965.542 | -30.0409 | 327-334 | (R)AYILKDSR(G) | |
| 970.619 | 970.6413 | -23.0012 | 281-288 | (R)KLVNKIQK(L) | |
| 1032.578 | 1032.5155 | 60.5738 | 362-369 | (R)VYFSVFDR(G) | |
| 1088.598 | 1088.5376 | 55.4426 | 127-136 | (K)TFSITYGSGR(M) | |
| 1178.673 | 1178.6646 | 7.1432 | 327-336 | (R)AYILKDSRGR(C) | |
| 1201.61 | 1201.6152 | -4.3171 | 337-345 | (R)CYTAFKKQR(F) | |
| 1389.723 | 1389.6585 | 46.4078 | 216-228 | (R)EGSVVMFGGVDHR(Y) | |
| 1405.714 | 1405.6534 | 43.0948 | 216-228 | (R)EGSVVMFGGVDHR(Y) | 1Met-ox |
| 1733.952 | 1733.8836 | 39.4634 | 113-126 | (R)FRQHQSSTFRPTNK(T) | |
| 1830.003 | 1829.9074 | 52.2487 | 346-361 | (R)FSSSTETWLLGDAFLR(V) | |
| 1860.005 | 1859.875 | 69.8736 | 216-231 | (R)EGSVVMFGGVDHRYYK(G) | 1Met-ox |
| 1999.099 | 1998.9925 | 53.2686 | 232-248 | (K)GELNWVPLIEEGDWSVR(M) | |
| 2153.122 | 2153.0327 | 41.4774 | 30-47 | (K)TLSGKNMLNNFLKEHGNR(L) | 1PO4 |

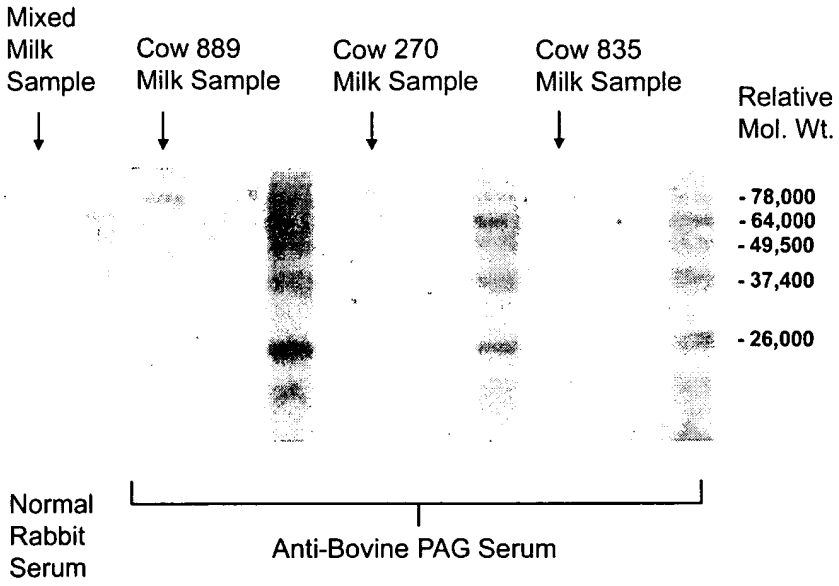
Weaker matches were observed for PAG-17, PAG-16 and PAG-20


63
BoPAG7 (NM 176618)
75
BoPAG16 (NM 176625)
A6 Antibody
50
BoPAG20 (AF192337)
J2 Antibody
BoPAG14 (AF192331)
BoPAG6 (NM 176617)
97
BoPAG4 (NM 176615)
A6 Antibody
97
BoPAG15 (NM 176624)
BoPAG19 (AF192336)
70
BoPAG21 (AF192338)
L4 Antibody
BoPAG1 (M73962)
97
BoPAG3 (L06153)
BoPAG9 (NM 176620)
100
BoPAG18 (AF192335)
53
BoPAG17 (AF192334)
A6 and L4 Antibodies
BoPAG5 (NM 176616)
99
BoPAG10 (NM 176621)
BoPAG8 (NM 176619)
BoPAG11 (NM 176623)
BoPAG12 (NM 176622)
BoPAG2 (NM 176614)
100
100
BoPAG13 (AF192330)
0.05

**Concentration of PAG in serum, milk and urine**

| | SERUM | MILK | URINE |
|---|---|---|---|
| Polyclonal Ab ELISA | | | |
| Cow 889 (Open -18 days postpartum) * | 380ng/mL | 170ng/mL | 13.5ng/mL |
| Cow 898 (Open -34 days postpartum) | 110ng/mL | undetectable | undetectable |
| Cow 788 (Open - 24 days postpartum) | 555ng/ml | undetectable | 12ng/mL |
| Cow 270 (pregnant) | 480ng/mL | 49ng/mL | 17ng/mL |
| Cow 848 (pregnant) | 160ng/mL | undetectable | undetectable |
| Cow 835 (pregnant) | 850ng/mL | 6.25ng/mL | 60ng/mL |
| Monoclonal Ab ELISA | | | |
| Cow 889 (Open -18 days postpartum) * | undetectable | 12.5ng/mL | undetectable |
| Cow 898 (Open -34 days postpartum) | undetectable | undetectable | undetectable |
| Cow 788 (Open - 24 days postpartum) | 25ng/mL | undetectable | undetectable |
| Cow 270 (pregnant) | 95ng/mL | 6.5ng/mL | undetectable |
| Cow 848 (pregnant) | 12.5ng/mL | undetectable | undetectable |
| Cow 835 (pregnant) | 450ng/mL | 1.5ng/mL | undetectable |

* Cow 889 had mastitis


Mixed
Milk
Sample
Cow 889
Milk Sample
Cow 270
Milk Sample
Cow 835
Milk Sample
Relative
Mol. Wt.
- 78,000
- 64,000
- 49,500
- 37,400
- 26,000
Normal
Rabbit
Serum
Anti-Bovine PAG Serum